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(54) Title: PEPTIDES ENDOWED WITH ANTIINFLAMMATORY ACTIVITY

(57) Abstract

Peptides consisting of 25 aminoacids having a sequence with an h omology of at least 25 % with the 1-25 fragment of the 10 Kda heat shock protein from Mycobacterium tubercolosis are disclosed. The peptides of the invention are endowed with antiinflammatory activity.

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PEPTIDES ENDOWED WITH ANTIINFLAMMATORY ACTIVITY

The present invention relates to peptide derivatives of heat shock proteins, and to the use thereof in the treatment of inflammatory pathologies.

The heat shock proteins (hereinfrom "HSP") are produced by cells under stress conditions, especially by mycobacteria. Procaryotes such as mycobacteria, express high HSP levels, some of which, e.g. a 65 kD protein, are immuno-dominant antigens, thus their use as vaccine was envisaged, e.g. antitubercolotic vaccine [(Kaufmann, S.H.E. et al., Eur. J. Immunol., 17, 351 (1987)]. WO 89/12455 gives a hint about the use of a protein of such class or a fragment thereof, specifically referring to a 65 kD protein, as a vaccine against non-viral infections and to induce an immune response.

Specific proteins within the same class were described as useful in different pathologies. For example, WO 90/10449 relates to the use of a HSP of 65 kD as a diagnostic agent and in the treatment of the insulin-independent diabetes. The same protein was found to posses a mycobacterial-specific epitope envolved in the pathogenesis of the auto-immune arthritis [Gaston, J.F. et al., Nature, 331, 171 (1988)].

The HSP sequence weighing 10 kD is disclosed by Baird, P.N. et al., J. Gen. Microb., 135, 931-939 (1989) which describes it as coming from Mycobacterium tubercolosis BGC, while Mehra, V. et al., J. Exp. Med., 175, 275-284 (1992) discloses a homologous protein having the same weight from Mycobacterium lepræ. Barnes, P.F. et al., J. Immun., 148, 1835-1840 (1992) discloses

a 10 kD protein coming from Mycobacterium tubercolosis as highly immuno-reactive antigen hypothetically useful as antitubercolotic vaccine. Hartman, D.J. et al., Proc. Natl. Acad. Sci. USA, 89, 3394-3398 (1992) identified, in the mammal, a protein homologous to the 10 kD proteins described in the literature above mentioned.

It has been now found that peptides of 25 amino acids having a sequence corresponding to or with a homology of at least 25% with the 1-25 fragment of the HSP from Mycobacterium tubercolosis, are endowed with antiinflammatory activity.

Therefore, the invention relates to a peptide of 25 amino acids having a homology ≥ 25% with the following amino acid sequence I (Sequence Id No. 1):

NH_2-Ala Lys Val Asn Ile Lys Pro Leu Glu Asp Lys Ile Leu Val Gln Ala Asn Glu Ala Glu Thr Thr Thr Ala Ser-OH wherein the N-terminus is optionally acylated.

It is particularly preferred a 1-25 peptide having the amino acid sequence I.

Said peptides are useful in the treatment of inflammatory pathologies, especially in the treatment of rheumatoid arthritis.

The peptides of the invention are prepared by conventional chemical methods of peptide synthesis. A method is the one in solid phase originally developed by Merrifield, R.B. (Biochemistry 1964, 3, page 1385; The Peptide 1979, 2, page 1-284, E. Gross and J. Meienhofer Ed.). Alternatively, the synthesis may be carried out, always in solid phase, applying the flow method and using Fmoc-amino acids optionally protected on the side-chain by acid-labile groups [Atherton E. and Sheppard

R.C., "Solid phase peptide synthesis - a practical approach", IRL PRESS, Oxford, 1989]. In the latter case a commercially available automatic or semiautomatic synthetizer (e.g., Milligen^R 9050) is used, and the solid support may be one of the resins suitable to this synthetic method (e.g., NovaSyn^R resins of Novabiochem, or PepSyn^R resins of Milligen KA). Usually, these resins contain norleucine residues (as internal reference amino acid) to which the reversible anchoring agent for the peptide to be prepared may then be linked. The anchorage agent may be, for example, p-hydroxymethyl-phenoxyacetic acid. In this case, among the commercially available resins, the ones just containing the protected derivative of the first amino acid linked by an ester bond to the resin may be employed.

Generally, in any case the peptide synthesis is carried out through a series of deprotection cycles with 20% piridine in dimethylformamide (DMF), repeated short washings with DMF, acylation and again repeated washings with DMF, according to the standard procedures provided by the synthetizer manufacturer and the modifications thereof obvious to the skilled in the art, which are automatically performed by the apparatus. The single protected amino acids are used as activated esters to assemble the peptide, such ester being pre-formed and commercially available, or prepared in situ without isolation, for example as phenolic esters or as 1-hydroxy-benzotriazol or 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine esters or analogues thereof. Actually, the suitably protected amino acid is reacted with a condensing agent such as, for example, di-

cycloalkyl-carbodiimide, di alkyl-carbodiimide or benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) and analogues thereof, in the presence of the selected phenolic derivative such as, e.g. pentafluorophenol or of 1-hydroxy-benzotriazole (HOBT) or 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine (HODhbt). For each condensation a 4 times excess with respect to the amino groups was used. At the end of the synthesis, the peptide may be removed from the resin by means of one of the protocols known to the skilled in the art. For example, 0.5 g of resin+peptide suspended in about 10 ml of a mixture of 90% trifluoroacetic acid (TFA), 5% thioanisole, 3% ethandithiole, 2% anisole, is kept at room temperature, under mild stirring and under nitrogen for 4 hours. The mixture is then directly filtered in a 10-20 times bigger volume of ethyl ether cooled in ice-bath. The precipitate is filtered or centrifuged, then dried under vacuum overnight. The peptide is dissolved in a suitable buffer and freeze-dried. Another method implies the suspension of 0.5 g of resin+peptide in about 25 ml of a mixture of 1M trimethyl-silyl-bromide (Me3SiBr) 1M thioanisole, 0.25M ethandiole in trifluoroacetic acid, and maintaining the whole at 0°C under mild stirring and under nitrogen for 1 hour. The resin is then filtered and washed with a small volume of pure TFA. The solvent is evaporated, and the residue tritured in ethyl ether is filtered or centrifuged, then dried under vacuum overnight. The peptide is dissolved in a suitable buffer and freeze-dried.

The following example better illustrates the

invention.

EXAMPLE

Sinthesis of the 1-25 fragment of HSP-10 from Mycobacterium tubercolosis

Sequence: H-Ala Lys Val Asn Ile Lys Pro Leu Glu Asp Lys Ile Leu Val Gln Ala Asn Glu Ala Glu Thr Thr Thr Ala Ser-OH

The solid support [1 g of Fmoc-Ser(tBu)-PepSyn KA (100); resin substitution 0.09 mmol/g] was charged on the column of a Milligen^{R} 9050 synthetizer and submitted to a standard series of deprotection and acylation cycles. Each single amino acid residue employed had the α-amino group protected with Fmoc, whereas the protecting group of the side-chains were tert-butyloxycarbonyl (Boc) for lysine, tert-butyl (tBu) for aspartic and glutamic acid, serine and threonine. All of the so protected amino acids were pre-activated as pentafluorophenol ester excepting for serine and threonine, pre-activated as HODhbt esters. Each single residue was sequentially assembled (in a 4 times molar ex cess) starting from the C-terminus amino acid, through single and/or double coupling cycle in about 60 minutes. The final cleavage of the peptide from the resin and the detachment of the protecting groups from the side-chains were effected on a scale of 0.5 g of peptide-resin following one of the protocols above described. After freeze-drying, there were obtained 100 mg of crude peptide (molecolar weight = 2684; calculated yield: 120 mg), yield 83%. 50 mg were charged on a semi-preparative reversed-phase column (Vydac C4, 25x1 cm), balanced with eluent A) 0.085% TFA in water, and eluted

with eluent B) 0.085 TFA in acetonitrile:water 80:20, applying a gradient of 0.27% B/minute at a flow of 3.0 ml/minute. There were thus obtained 13 mg a product with a final yield over the crude of 26%. The relative purity of the peptide was determined by HPLC analysis on a reversed-phase Vydac C4 column (150x4.6 mm), using as eluent A) 0.045% TFA in water:acetonitrile (98:2 v/v) and as eluent B) 0.036% TFA in acetonitrile, with a gradient of 2% B/minute.

The amino acid composition of the peptide (Tab. 1) was determined by an amino acid BECKMAN System Gold 126 AA analyzer, after hydrolysis at 110°C for 22 hours in 6N HCl in the presence of 1% phenol v/v, in sealed phials under vacuum: peptide content 88%. The molecolar weight of the peptide was determined by mass spectrometry (BIOMASS spectrometer, ELECTRO-SPRAY ionizer, quadrupole, accuracy 0.05-0.01%): calculated 2684; found 2684.

TABLE 1

Amino acid	calculated	found
Asp/Asn	3	3.01
Thr	3	2.92
Ser	1	0.63
Glu/Gln	4	3.85
Pro	1	1.03
Ala	4	3.99
Val	2	1.96
Ile	2	1.87
Leu	2	2.03
Lys	3	3.09

The peptide of the present invention are useful in

the treatment of inflammatory pathologies of different kind and origin, as it is shown by pharmacological tests (adjuvant arthritis test) as follows.

15 Wistar rats (C. River; weight 130-140 g) and anaesthetized with CO_2, were intradermically administered (injection at the base of the tie) with 0.1 ml of a suspension of 10 mg/ml of heat-inactivated M. tuberculosis (Strain C, DT and PN; Central Vet. Labs - GB), in sterile paraffin oil. The rats were divided in 3 groups of 5 animals each, and at day 4, 5 and 6 from the above treatment, following the same method for inducing arthritis, they were administered with 50 µg/rat dose of the peptide I in 100 µl of PBS for the first group, with PBS only for the second group, while the third group was not treated. The course of the arthritis was monitored according to the following scheme of clinical scores:

score	symptomatology
0	no inflammation
1	slight redness and swelling of the paws
2	swelling of the paws such that the tendons are no longer visible
3	swelling extending to the ankle joint
4	marked inflammation and deformity of the ankle joint

The scores range from 0 to 4 for each paw; furthermore one additional score is assigned if there are nodules on the tie, and another futher score is assigned if ears are involved, thereby the score is 0 at minimum and 18 at maximum.

The results are set forth in Tables 2 and 3.

TABLE 2

Treatment	Clinical scores (± S.E.)					
	day 7	day 8	day 10	day 11	day 12	day 13
1-25	0	0	0.6±0.6	3.8±0.4	10.0±0.7	12.6±0.6
PBS	1.4±0.2	1.8±0.4	7.4±1.3	11.6±0.6	14.8±0.4	15.6±0.6
Control	0.2±0.2	1.4±0.4	5.8±1.1	9.4±0.7	13.4±1.2	15.4±0.6

TABLE 3

Treatment	Incidence of arthritis (arthritic rats/total rats)				
	day 7	day 8	day 10	day 11	day 12
1-25	0/0	0/0	1/5	5/5	5/5
PBS	5/5	5/5	5/5	5/5	5/5
Control	1/5	4/5	5/5	5/5	5/5

Object of the present invention is therefore the use above mentioned peptides in the treatment of inflammatory pathologies, referring to all the industrial aspects connected to said use also including their incorporation into pharmaceutical compositions. For the envisaged pharmaceutical uses, the peptides of the invention may be administered suitably formulated into pharmaceutical compositions for parenteral administration, particularly intradermically, subcutaneously and intra-articularly injectable formulations. As for the intradermically and subcutaneously injectable formulations, the active principle may be dissolved in bidistilled water, optionally in the presence of isotonic agents such as dextrose or sodium chloride, antimicrobials such as p-hydroxy-benzoates, and buffers, for example a phosphate buffer such as PBS. As for the intra-articularly injectable formulations, it is necessary the presence of an isotonic agent such as one of the already above said, together with the other just mentioned excipients. The active principle may also be formulated as a restorable freeze-dried product containing from 4 to 8% of mannitol or lactose. Obviously the posology depends from various parameters such as the kind and severity of the pahologies to be treated, and the conditions of the patient (weight, sex, age, etc.).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: ITALFARMACO S.P.A.
(B) STREET: Via Carducci 125
(C) CITY: Sesto San Giovanni
(D) STATE: Milan
(E) COUNTRY: Italy
(F) POSTAL CODE (ZIP): 20099

(ii) TITLE OF INVENTION: PEPTIDES ENDOWED WITH ANTIINFLAMMATORY ACTIVITY

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Mycobacterium tuberculosis

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ala Lys Val Asn Ile Lys Pro Leu Glu Asp Lys Ile Leu Val Gln Ala
1 5 10 15

Asn Glu Ala Glu Thr Thr Thr Ala Ser
20 25

CLAIMS

1. Peptides consisting of 25 amino acids having a homology of at least 25% for the following sequence I:
NH_2-Ala Lys Val Asn Ile Lys Pro Leu Glu Asp Lys Ile Leu Val Gln Ala Asn Glu Ala Glu Thr Thr Thr Ala Ser-OH (I) being said sequence optionally acylated at the N-terminus.

2. A peptide according to claim 1 having the amino acid sequence I.

3. Pharmaceutical compositions containing as active principle a peptide of claims 1 or 2 together with a suitable carrier.

4. Use of a peptide according to claims 1 or 2 for the preparation of a medicament for treating inflammatory pathologies.

5. Use of a peptide according to claims 1 or 2 for the preparation of a medicament for treating rheumatoid arthritis.

INTERNATIONAL SEARCH REPORT

Interr ·al Application No
PCT/EP 95/04566

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/35 A61K39/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GB,A,2 251 186 (R.N.GATZ ET AL) 1 July 1992 see the whole document ---	1-5
A	WO,A,92 04049 (UNIV UTRECHT ;YEDA RES & DEV (IL); NEDERLANDEN STAAT (NL)) 19 March 1992 see the whole document ---	1-5
A	WO,A,89 12455 (WHITEHEAD BIOMEDICAL INST ;MEDICAL RES COUNCIL (GB)) 28 December 1989 cited in the application see the whole document ---	1-5
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
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Interr ·al Application No
PCT/EP 95/04566

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 89, April 1992 WASHINGTON US, pages 3394-3398, D.J.HARTMAN 'Identification of a mammalian 10 kDa HSP ...' ---	1-5
A	WO,A,87 01118 (SCRIPPS CLINIC RES) 26 February 1987 see claims 1-40 -----	1-3

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INTERNATIONAL SEARCH REPORT

formation on patent family members

Interr ·al Application No
PCT/EP 95/04566

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
GB-A-2251186	01-07-92	NONE		
WO-A-9204049	19-03-92	AU-B-	650065	09-06-94
		AU-B-	8755991	30-03-92
		CA-A-	2071896	07-03-92
		EP-A-	0503055	16-09-92
WO-A-8912455	28-12-89	AT-T-	127345	15-09-95
		DE-D-	68924162	12-10-95
		EP-A-	0419569	03-04-91
WO-A-8701118	26-02-87	US-A-	4689397	25-08-87
		AU-B-	605108	10-01-91
		AU-B-	6229386	10-03-87
		CA-A-	1306582	18-08-92
		DE-A-	3688246	13-05-93
		DE-T-	3688246	21-10-93
		EP-A,B	0233936	02-09-87
		JP-A-	7300497	14-11-95
		JP-B-	7062030	05-07-95
		JP-T-	63500524	25-02-88
		US-A-	4889800	26-12-89